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14. ABSTRACT
DNA damage in the form of double-strand breaks is caused by exposure to endogenous factors as well as in response to radiation therapy in breast cancer patients. Double-strand breaks can be repaired by homologous recombination or non-homologous end joining pathways, both of which can lead to error-prone repair. Errors in repair lead to accumulation of mutations that may accelerate the process of tumorigenesis and malignant transformation. Apart from cell cycle effects, little is known about which factors contribute to the determination of double-strand break efficiency or pathway choices in mammalian cells. We previously showed that extracellular matrix signaling can regulate double-strand break repair pathway choice in a human breast epithelial cell line. Furthermore, we find that the kinetics of foci formation by a DNA damage signaling protein after ionizing radiation is altered by extracellular matrix signaling. Here we found that in primary mouse mammary epithelial cells extracellular matrix signaling regulates double-strand repair, as well as the kinetics of damage signaling after ionizing radiation. Having shown that the effects of ECM on DNA damage signaling and repair are generalizable, we will use mouse models with altered extracellular matrix signaling or DNA repair components to genetically dissect this pathway in vivo.

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INTRODUCTION

DNA damage in the form of double-strand breaks (DSBs) is experienced by normal breast cells via exposure to endogenous factors, such as estrogen (Li et al., 1994) and the stalled replication forks during replication(Michel et al., 1997), as well as in response to radiation therapy in breast cancer patients. DSBs can be repaired by homologous recombination (HR) or non-homologous end joining (NHEJ) pathways, both of which can lead to error-prone repair depending on genomic location, whether or not repeat sequences are involved, and the sub-pathways used in repair (Mills et al., 2003). Error-free and efficient repair contribute to maintenance of genomic stability, while the loss of genome stability leads to accumulation of mutations and may accelerate the process of tumorigenesis and malignant transformation (Mills et al., 2003). Therefore, regulation of double-strand break repair (DSBR) efficiency and pathway choice (HR vs. NHEJ) has important consequences in maintaining a normal breast epithelium. Apart from cell cycle effects, little is known about which factors contribute to the determination of DSBR efficiency or pathway choices in mammalian cells. We propose a new concept that the extracellular matrix (ECM), which is found in the basement membrane surrounding breast epithelial ducts and acini, can regulate DSBR efficiency and pathway choice, thereby maintaining genomic stability. This then predicts that loss of BM integrity, which is evident in all breast cancers, would result in error-prone repair that is conducive to further mutagenesis.

BODY

Previously, our exploratory experiments indicated that ECM can regulate DSBR pathway choice in a non-tumorigenic human breast epithelial cell line (HMT3522-S1) as measured by the HR of a single I-SceI endonuclease induced DSB within a direct repeat substrate. This regulation was independent of cell cycle effects and was mediated by β1 integrin receptor signaling. Furthermore, we found that the kinetics of repair of DSBs induced by ionizing radiation is altered by ECM, as determined by quantification of phosphorylated H2AX foci as a measure of DSBs. Encouraged by these novel observations in a human breast epithelial cell line, we proposed to explore whether ECM signaling regulates DSBR in the mouse mammary epithelial cells (mecs) in primary cultures. Our main objectives were as follows:

1.Determine if ECM and β1 integrin regulate HR of an endonuclease-induced break in mecs
2.Determine if β1 integrin-mediated signaling alters repair kinetics of ionizing radiation-induced breaks in mecs

To achieve these objectives we proposed the following tasks in our Statement of Work and made progress towards achieving these as follows:

***Task 1.* Determine if ECM and β1 integrin regulate HR of an I-SceI induced break in the mouse mammary gland (Months 1-6).**

a. Obtain sufficient numbers of transgenic mice that are female and are homozygous for the DR-GFP substrate to allow isolation of a large number of mammary glands that will be needed. The number of animals needed will depend on the actual frequency of HR in these cultures and will have to be empirically determined. Mammary glands will be frozen until the required number has been obtained to perform one experiment. We have developed methods that allow us to establish primary cultures from frozen mammary glands successfully (Months 1-3).

Using a total of 345 mice (305 at LBNL and 40 at MSKCC), we bred and collected mammary glands from either heterozygote DR-GFP (+/-) or wildtype (-/-) female mice at 12 weeks of age. Glands from a total of 140 heterozygote and 59 wildtype female mice were frozen. 104 heterozygote and 18 wildtype mice glands were used in experiments. We continue to breed the DR-GFP mice to obtain sufficient tissue for ongoing and future experiments (Figure 1).

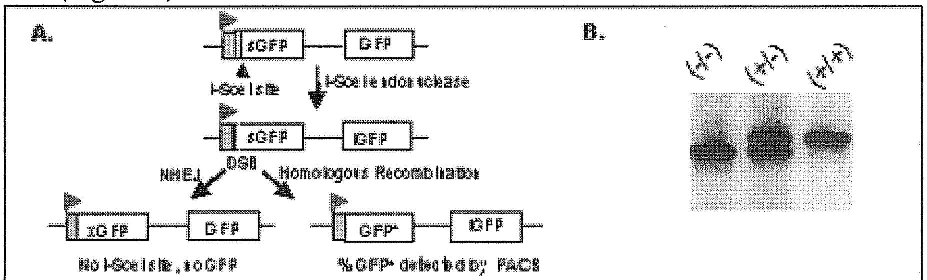

Figure 1. The homologous recombination (HR) assay in DR-GFP mice.
A. The DR-GFP substrate contains two non-functional GFP genes in tandem. The first GFP (left) has an 18bp I-SceI endonuclease site that renders it mutated, the second GFP has no promoter and sequence deletion on its 5' end. A double-strand break (DSB) is produced when I-SceI endonuclease is introduced into the cell. Repair of the DSB by either sister chromatid exchange or intrachromosomal recombination (HR) results in reconstitution of a functional GFP sequence. GFP positive cell proportion is determined by FACS analysis as an indication of HR frequency. **B.** Diagnostic PCR for the PIM1 gene into which the DR-GFP was targeted in transgenic mice, showing DRGFP heterozygous (+/-), homozygous (+/+), and wildtype (-/-).

b. Produce sufficient lentivirus for all experiments, using the I-SceI and the control GFP vectors. We find that lentiviral yields are usually low and many large scale experiments are needed to obtain amounts that will be needed for each experiments, given that a large number of cells will have to be infected because HR frequencies are low and we would need to FACS sort sufficient number of cells to reliably and reproducibly detect the GFP positive portion (Months 1-3)

We have been producing and concentrating lentiviruses expressing the I-SceI and the GFP control genes in sufficient amounts to use in our HR experiments, using the common method of ultracentrifugation to concentrate the virus 50 fold prior to use. We obtained a minimum infection efficiency of about 10% in the presence of ECM (5% Matrigel). The fact that we were unable to obtain the 50% efficiency we had anticipated meant that we required a higher number of mice to perform the experiments we proposed and we were unable to complete all proposed mouse breedings required within the time frame of the proposal.

c. Titrate the β1-integrin blocking antibody concentrations to determine how much is needed to dowregulate integrin activation under the primary culture conditions, by determining downstream signaling components by Western analysis (Months 1-3).

We discovered that our colleagues in the laboratory had already done this for a mouse cell line, and used a concentration of 10μg/mL for our experiments as well.

d. Determine how many days are needed to complete DSBR after I-SceI is introduced into the primary cultures in simple monolayer cultures of homozygous DR-GFP mammary glands. Add a few days to this number to ensure that repair will be complete under various culture conditions. (Months 1-6)

We found that 5 days is sufficient for the formation of the maximum number of GFP positive cells in the culture as a result of HR. Trypsinization and replating of cells after 7 days in culture did not increase the frequency of HR. We allowed 7 days after lentiviral introduction of the endonuclease to compare the effects of ECM on HR.

e. Compare HR among lrBM plus/minus, β1-integrin-blocking-antibody plus/minus culture conditions. Parallel cultures will be needed to ensure that growth is inhibited as intended, by determining BrDU and Ki67 marker expression in parallel cultures to the experimental ones (Months 1-6)

We compared HR between dividing (+serum) and non-dividing (-serum) cultures in the absence of ECM and found there to be no difference (1.74 +/- 0.32 vs 1.72 +/-0.28 %). This could be explained by the fact that the substrate we used in these experiments is a direct repeat substrate which allowed HR to occur intrachromosomally in the absence of a sister chromatid in non-dividing cells as well. These results are consistent with what we observed in the human S1 cell line as well. Given this observation, we proceeded to determine the effect of ECM on HR using serum containing cultures in order to obtain sufficiently high numbers of GFP positive cells to produce statistically significant results when cells were analyzed by FACS. We found that ECM upregulates HR in dividing primary mammary epithelial cell cultures (Figure 2).

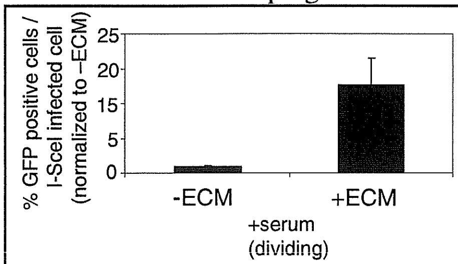

Figure 2. ECM upregulates HR in primary mouse mecs.
Organoids containing the epithelial cells of the breast tissue were isolated from heterozygote DR-GFP mice mammary glands. These were plated in the presence of serum, allowed to attach and I-SceI endonuclease or control GFP expressing lentiviruses infected these, either in the presence or absence of ECM After 7 days of I-SceI break induction and repair in culture in the presence or absence of ECM, cells were trypsinized and the proportion of GFP positive cells in I-SceI transduced cultures was determined as an indicator of HR. This number was corrected to the infection efficiency under each culture condition, in parallel cultures transduced with lentiviral GFP. The HR frequency determined in this manner is shown in the grapth, represents results from 4 separate experiments, and is normalized to the –ECM HR values to allow combining results across experiments.

Parallel to the experiments for HR described above, we determined Ki67% during the 7 day experiment, using wildtype gland cultures (Figure 3). BrDU experiments are in progress.

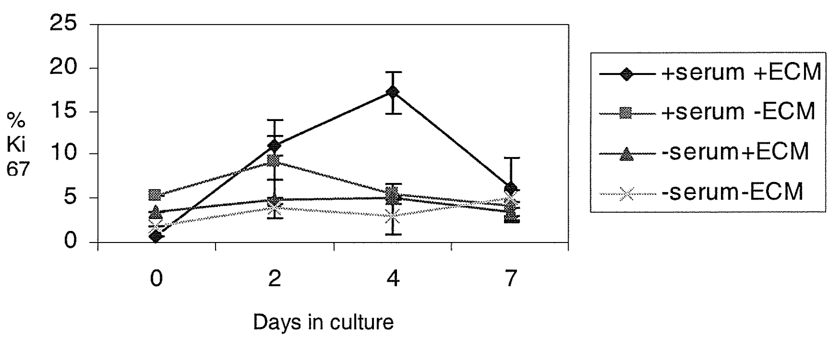

Figure 3. Ki67 positivity during the HR experiment.
Wildtype organoids were plated under the conditions for serum and ECM as indicated. Cells were immunostained with Ki67 at the indicated time points after plating, and the % Ki67 determined against total number of cell nuclei stained with DAPI. Data from two experiments is plotted. Note: there was no difference in HR between (+serum –ECM, and –serum –ECM conditions; HR was upregulated in +serum +ECM, compared to +serum –ECM, in parallel experiments)

Experiments to determine whether the ECM effect on HR is β1 integrin dependent will need to be performed using non-dividing (-serum) cultures because of the known effects of β1 integrin on growth. We continue to collect glands to complete these experiments using the known Ha2/5 monoclonal antibody concentration (also used in foci experiments below) to block β1 integrin function.

***Task 2.* Determine if β1 integrin-mediated signaling alters the repair kinetics of ionizing radiation induced breaks in the mouse mammary gland (Months 6-12).**

a. Perform mouse crosses to obtain sufficient numbers of female mice homozygous for the floxed β1-integrin transgene (Months 6-9)

b. Produce sufficient amounts of Cre recombinase expressing lentivirus, as well as control GFP expressing virus which will be used as a control of infection efficiency under different culture conditions (Months 6-9)

c. Determine how much Cre recombinase expressing lentivirus will be needed to excise β1-integrin under different culture conditions. We know we can deliver Cre recombinase and knockout β1-integrin conditionally using adenoviral delivery. We also know that we can deliver lentiviral GFP control virus efficiently to primary mammary organoids under the proposed culture conditions. However, we have yet to determine how much lentiviral Cre recombinase can be used to knockout β1-integrin without compromising cell viability. It is crucial that we do not use adenovirus since adenoviral infection has been reported to alter DSBR pathway component levels (Months 6-9)

d. Determine the repair kinetics of ionizing radiation (X-ray) induced breaks using gamma-H2AX foci numbers as an endpoint. A range of doses from low doses such as 0.1 and 1 Gy, to high doses such as 3 and 6 Gy will be used. We will need to control for growth as well, and to measure growth using BrDU and Ki67 in parallel cultures. Repair kinetics of β1-integrin knocked out cells will be compared to the β1-integrin expressing cells, in the presence or absence of lrBM. (Months 10-12)

Given the lower than expected lentiviral transfection efficiency, before launching into breeding the large number of β1 integrin floxed mice for controlled homozygous deletion of β1 integrin in culture using cre recombinase, we first used the β1 function blocking antibody Ha2/5 in order to determine if dowregulating β1 integrin function alters the kinetics of gamma-H2AX foci formation in primary mammary epithelial cell cultures. We found that in non-dividing (-serum) cultures, ECM addition to the culture increased the number of foci formed in response to 3Gy irradiation, and that this increase was reversed by blocking β1 integrin (Figure 4). This increase is consistent with the upregulation effect of ECM on HR as well.

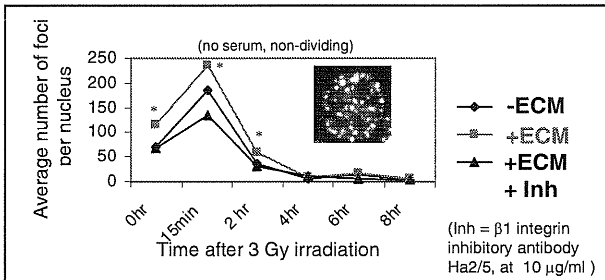

Figure 4. gamma-H2AX foci kinetics in primary mouse mammary organoids. Average number of foci per nucleus was determined after 3Gy irradiation for a minimum of 50 nuclei in a minimum of 3 cultures. 2μm confocal sections of γ-H2AX immunostained nuclei were counted manually. +Inh, β1 integrin inhibitory antibody Ha2/5 at 10μg/ml. *, $p<0.05$ for compared to "No ECM". Inset shows sample foci within one nucleus.

Our goal is to strengthen this result by asking whether complete abrogation of β1 integrin would have a more striking effect than the partial function blocking using the monoclonal antibody. The results we obtained here provide us with a good rationale for the floxed β1 integrin mouse breeding experiments, which are in progress.

KEY RESEARCH ACCOMPLISHMENTS

- We determined that ECM signaling upregulates the homologous recombination repair of a double-strand break within a direct-repeat substrate in primary mouse mammary epithelial cells. This result provides us with a rationale to design experiments to test the effect of ECM signaling on double-strand break repair in the mouse mammary gland in vivo.

- We found that ECM signaling via β1 integrin upregulates double-strand break induced foci formation in response to ionizing radiation. This is consistent with the effect of ECM on homologous recombinational repair and provides us with a rationale to explore the effects of ECM on radiation induced double-strand breaks in the mouse mammary gland in vivo.

REPORTABLE OUTCOMES

- The results were incorporated into a manuscript in preparation which also described the human cell line data (Rizki et al., In preparation), to provide evidence for the generalizability of the effects of ECM on double-strand break repair.

- The Concept Award provided funding for the PI to attend the Mutagenesis Gordon Conference in Newport, RI in August 2006 where a poster titled "Extracellular Matrix Signaling Regulates Double-strand Break Repair" was presented.

- The data generated here were used in an NIH K99/R00 Pathway to Independence Award application by the PI (pending).

- The PI who is at the end of her postdoctoral training used the data in proposing future experiments to be performed as an independent faculty member in a future hiring institution. ~150 faculty job applications were generated which used the proposed future goals. These applications have so far resulted in 10 interview offers and all except 30 of them are still pending. The results generated have so far been presented at the Fox Chase Cancer Center, Roswell Park Cancer Research Institute, the Department of Toxicology at University of California at Santa Cruz, and the Department of Biochemistry, Molecular Biology, and Cell Biology at Northwestern University, and will continue to be presented in the remaining faculty job seminars.

- The ability to obtain an independent grant via a funding mechanism which encourages creativity, i.e. the Concept Award, was used as important supporting evidence in an Immigrant Petition based on National Interest Waiver, which was recently granted. This will enhance the ability of the PI to continue permanently residing and doing science in the U.S.

CONCLUSIONS

This award allowed us to explore the effects of ECM signaling on DNA double-strand break repair in primary cultures of mouse mammary epithelial cells. This was important to do because we had previously used a human cell line and found that ECM signaling via β1 integrin downregulated both the homologous recombinational repair of an endonuclease-induced break within a direct-repeat substrate, and the formation of ionizing radiation induced gamma-H2AX foci, suggesting that ECM signaling regulates both double-strand break repair pathway choices and the DNA damage signaling pathways induced. Whether this novel effect of ECM on DNA damage signaling and repair applied to other cell systems, and more importantly, whether this also occurs in vivo were questions that needed to be answered.

Using primary mammary epithelial cell cultures, we found that ECM signaling upregulates homologous recombinational repair within a direct repeat. We also found that DNA damage signaling measured by formation of ionizing radiation induced gamma-H2AX foci (at sites of double-strand breaks) was upregulated by ECM signaling in a β1 integrin dependent manner. These results provide us with sufficient evidence that the effect of ECM on DNA damage signaling and repair are not specific to one human cell line and are generalizable to primary mouse cell cultures. We can now determine whether similar effects are observed in vivo and what the genetic determinants of this novel pathway are, using transgenic and knockout mouse models.

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